Directed Evolution of Enzymes for the Conversion of Corn-Fiber for Biofuel Production

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Summary

Currently, US ethanol fuel is mainly derived from grain (mostly corn) starch. The starch content is only about 71% of the corn kernel. The protein/fat and leftover fibrous materials (30-50% arabinoxylan, 15-20% cellulose and 10-25% residual starch), referred to as corn fiber, are processed into animal feed. Enzymatic saccharification is desirable as a biocompatible, environmentally responsible alternative. The major component of corn fiber, xylan, is a complex heteropolysaccharide with a β-1,4-xylose backbone rich in arabinose side chains. Endo-xylanase is the primary enzyme that attacks the backbone structure by randomly cleaving the internal xylosidic linkage, and the products are further hydrolyzed by β-xylosidase (exo-xylanase). However, the complex side-chain structures (mostly arabinose, together with glucuronic acid/ester and acetic, ferulic and coumeric acids) greatly reduce the accessibility of the backbone. Thus saccharification of corn fiber may require additional enzymes, including β-xylosidase, α-L-arabinofuranosidase, α-glucanronidase, acetyl xylan esterase and feruloyl esterase. We proposed to address these challenges by the application of directed protein evolution technologies. We also proposed the creation and evolution of an arabinofuranosidase/xylosidase fusion protein for increased activities on corresponding natural substrates. In the 12-month project period, we have fulfilled most of the proposed tasks and designed and performed additional experiments that further advanced the project. We have isolated additional genes encoding the xylosidase and arabinofuranosidase and expressed these genes in *E. coli* for recombinant protein productions. We have developed a number of high throughput assays that enable the screening of large numbers of enzyme variants. We have constructed and screened mutant libraries for the arabinofuranosidase; enzyme variants have been identified to potentially have xylosdiase activity. We have developed a capillary electrophoresis method to assay the hydrolases using natural substrate. We have also generated chimeric and trimeric fusion enzymes and characterized the

kinetic properties of these enzymes. Furthermore, we have transformed these fusion enzyme genes into tobacco plants to test the feasibility of producing these multifunctional proteins in transgenic plants. We have presented our results in two national conferences, as well as in the KREC project report meeting in 2006. The efforts supported by this GOEP seed grant have led to a grant from USDA/NRI awarded to the PI's laboratory.

Project Objectives:

(1) Use directed evolution technologies to improve the enzyme specificities and temperature optima of β-xylosidase and arabinofuranosidase, key enzymes for corn fiber degradation.

(2) Create a single enzyme with dual arabinofuranosidase/xylosidase activities in order to reduce the number of enzymes required for biomass conversion.

(3) Generate preliminary data for the application of additional full grants from federal agencies.

Progress Report:

1. Enzyme assay development

In our efforts to engineer hemicellulases with improved function, a high-throughput screening system is critical for the selection of variants from enzyme libraries. To this end we have established a number of sensitive assays using the *Thermoanearobacterium* xylosidase (GH39) and *Bacillus halodurans* arabinofuranosidase (GH51). More recently we have developed the following assays that will allow the screening of large numbers of variants:

Direct colony assay for xylosidase activity – To determine whether xylosidase activity can be assayed directly on isolated colonies, *E. coli* cells expressing the *Thermoanaerobacterium* xylosidase were mixed 1:1 with empty-vector control cells and grown on a nylon membrane overlaid onto LB medium. A replica plate was made using a velvet stamp. IPTG was added to the membrane for overnight induction of gene expression. The membrane was then lifted, submerged in lysis-buffer to release the expressed gene variants for the enzyme-coupled assay using xylobiose as substrate. In the assay xylosidase activity results in brown-colored precipitates. As show in Fig. 1, approximately 50% of the colonies on the membrane generated brown spots, indicating that the xylosidase activity can be detected from a single colony. This protocol will allow screening of large numbers of variants and is particularly useful for selecting

novel activity from an arabinofuranosidase library through the detection of brown spots. As an alternate strategy we have demonstrated the feasibility of picking libraries of gene variants into 384-well plates for use as master plates. Multiple master plates can then be arrayed onto membranes and processed to visualize active gene variants.

Figure 1. A portion of a replica plate on top of the membrane developed using xylobiose. Approximately half of the colonies developed brown color (one is indicated by the solid arrow) and the rest did not (for example see colony indicated by open arrow). Single colonies can be clearly distinguished.

Coupled solid-phase assays –His-tag purified xylosidase and arabinofuransidase were used in coupled solid-phase assays in 96-well format using xylobiose and xylotriose as substrates, and in liquid-phase assays using the chromophore tagged substrates *p*-NP-XylP and *p*-NP-AraF. Results indicate that the *Thermoanaerobacterium* xylosidase has low to no activity on p-NP-AraF but good activity on p-NP-XylP, xylobiose and xylotriose. Two additional arabinofuranosidases from *B. subtilis* and *B. halodurans* showed no activity toward p-NP-XylP, xylobiose and xylotriose, but good activity on p-NP-AraF. Figure 4 shows the enzyme-coupled assay using xylotriose as substrate in 96-well format. The assay has also been performed in 384-well format. These results indicate that the assay is suitable for high-throughput detection of novel arabinofuransidase substrate specificity to include xylosidase activity.

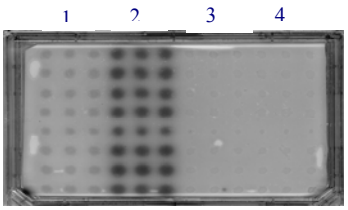


Figure 2. Solid-phase assays in 96-well format for xylosidase and arabinofuranosidase using xylotriose as substrate. The control and purified enzymes were transferred onto a nylon membrane in replica and incubated with the substrate. 1: empty-vector control; 2: *Thermoanearobacterium* xylosidase; 3: *B. subtilis* arabinofuransidase; 4: *B. halodurans* arabinofuranosidase.

2. Gene cloning and characterization of hemicellulases

We have cloned, expressed and characterized a large number of lignocellulosic enzymes from a variety of organisms. Recently, a 1581-bp gene encoding deAFc was isolated from a mixed bacterial culture (Dr. Earth compost starter) and expressed in *E. coli* (Wagschal et al. 2005. Appl Environ Microbiol 71: 5318). Protein sequence analysis revealed that deAFc is a member of the GH43 family. Gel-permeation chromatography indicated that the enzyme is a monomeric protein with an apparent molecular mass of 58 Kd. When synthetic substrates, p-nitrophyenyl-arabinofuranoside (p-NP-AraF), and o- and p-NP-xylopyranoside (o- and p-NP-XylP), were used, a broad pH profile with invariant V_{max} between pH5 and pH8.5 was observed (Fig. 3A). The enzyme was stable for 90 min up to 45^{o}C; however the activity decreased rapidly to ~10% following incubation for 5 min at 55 ^{o}C (Fig. 3B). The temperature maximum was determined to be 47 ^{o}C in a 30 min endpoint assay, whereupon activity rapidly diminished, presumably due to thermal instability.

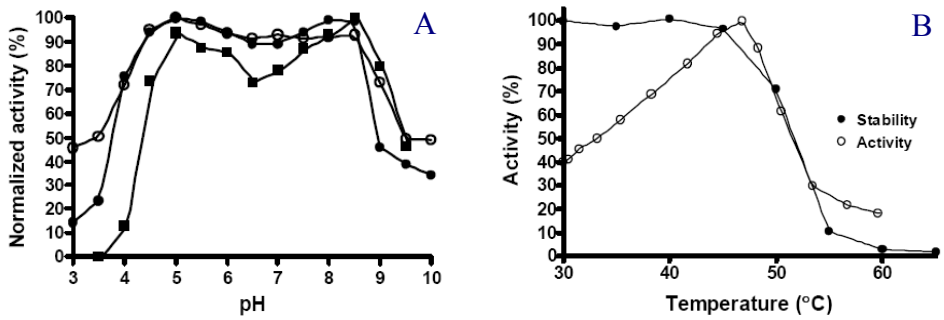


Figure 3. pH profiles and thermal activity/stability of deAFc. A: relative enzyme activity at the indicated pH values for substrates p-NP-AraF (•), p-NP-XylP (◦) and o-NP-XylP (■). B: relative enzyme stability (•) and activity (◦) at the indicated temperatures.

Enzyme kinetic parameters were also determined for deAFc using the synthetic substrates (Table 1). K_m values were of the same magnitude although the K_m for p-NP-AraF is 3 times lower than that for p-NP-XylP (250μM and 960μM, respectively). Interestingly, while the K_m values for

o- and p-NP-XylP were similar, the k_{cat} for o-NP-XylP is 9 times higher than that for p-NP-XylP (Table 1). We attribute the differential hydrolysis rates of o- and p-NP-XylP to the differences in noncovalent enzyme/substrate interactions (e.g. hydrogen bonding) of these steroelectronically different leaving groups in the active site and the attendant differences in stabilization of their respective oxocarbenium ion-like transition state. Nonetheless, deAFc clearly showed the greater k_{cat}/K_m for AraF.

Substrate	Concentration range (μM)	K_m (μM)	k_{cat} (sec^{-1})	k_{cat} / K_m ($mM^{-1} \bullet sec^{-1}$)
p-NP-AraF	30-4000	251 ± 11.8	0.684 ± 0.009	2.73 ± 0.133
p-NP-XylP	15-4000	960 ± 42.6	0.132 ± 0.003	0.138 ± 0.007
o-NP-XylP	30-7000	766 ± 41	1.22 ± 0.02	1.59 ± 0.09
4-MU-XylP [1]	10-4000	712 ± 66	0.571 ± 0.033	0.802 ± 0.088

[1] Results shown were obtained at pH 8.0. At pH 5.0 the kinetic parameters were K_m = 0.600 ± 54 μM, k_{cat} = 0.461 ± 0.025 sec^{-1} and k_{cat} / K_m = 0.768 ± 0.081. Reaction conditions were as described in the text.

Table1. Kinetic parameters of deAFc.

In order to accurately assign the substrate specificity of deAFc, a series of artificial and natural substrates were tested. In addition to hydrolysis of the artificial substrates reported above, release of arabinose, but not xylose, from rye, wheat and oat-spelt arabinoxylan was observed in TLC assays. The natural xylooligosaccharide substrate xylobiose was not hydrolyzed by deAFc. deAFc released arabinose from sugar beet arabinan containing (1-3)-α-linked L-arabinofuranosyl branch units; however, activity was not detected on the corresponding debranched 1,5-α-linked arabinan polymer. Therefore, our data indicate that deAFc is specific for hydrolyzing arabinofuranosyl units with preference for (1-3)-α-L-arabinofuranosyl branches.

3. Construction and screening of arabinofuranosidase DNA-shuffling labraries

Three arabinofuranosidase genes from *Geobacillus stearothermophilus*, *B. substili*s, and *B. haloduras* were used in DNA shuffling experiments for the construction and screening of mutants with xylosidase activity. The assembled PCR products were cloned into BamH I/Hind III sites of pET29b vector. The colonies with xylosidase activity were screened using a solid-phase assay (see above). A number of colonies showed increased xylosidase activity compared to the wild-type enzyme (Fig. 4). We are currently characterizing these enzyme variants.

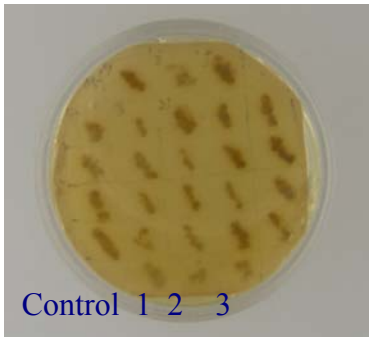


Figure 4. Arabinofuranosidase mutants with increased activity on xylobiose. A library was constructed by family DNA-shuffling and screened for xylosidase activity using a solid phase assay. Twenty three colonies were identified to have higher activity on xylobiose compared to the wild-type enzyme (control 1, 2, 3).

4. Generation of fusion enzymes

We have previously demonstrated that fusion enzymes can be generated for multi-step enzymatic reaction. A bacterial carotenoid biosynthetic gene (*crtI*) and a plant phytoene synthase (*psy*) have been used to create fusion proteins, linked by a poly-glycine peptide, for production of the antioxidant β-carotene. Reciprocal N-terminal fusions of the two enzymes (CrtI-psy and psy-CrtI) were generated. The fusion proteins have dual functions and are capable of catalyzing the two respective substrates, GGPP and phytoene, to produce carotene. The CrtI-psy fusion was determined to be more enzymatically active than the psy-CrtI fusion. The fusion protein has been expressed in *E. coli* and subjected to DNA shuffling as a single enzyme. The resulting library of

variants was screened visually by color intensity to identify variants with increased carotene production. Variants with increased activities up to 5-fold over the parent fusion enzyme were obtained. Transgenic rice expressing the evolved fusion crtI-psy protein accumulated significantly higher amounts of β-carotene than those co-expressing the two wild-type enzymes (L. Liu and L. Yuan unpublished results).

Creation of xylosidase-arabinofuranosidase chimera. The genes encoding the *Thermoanearobacterium* xylosidase and *B. halodurans* arabinofuranosidase were fused using a poly-glycine linker. The fusion gene was verified by sequencing and expressed in *E. coli*. The recombinant protein was characterized by SDS-PAGE and activity assays. The results indicate that the fusion protein is soluble and enzymatically active (Fig. 5).

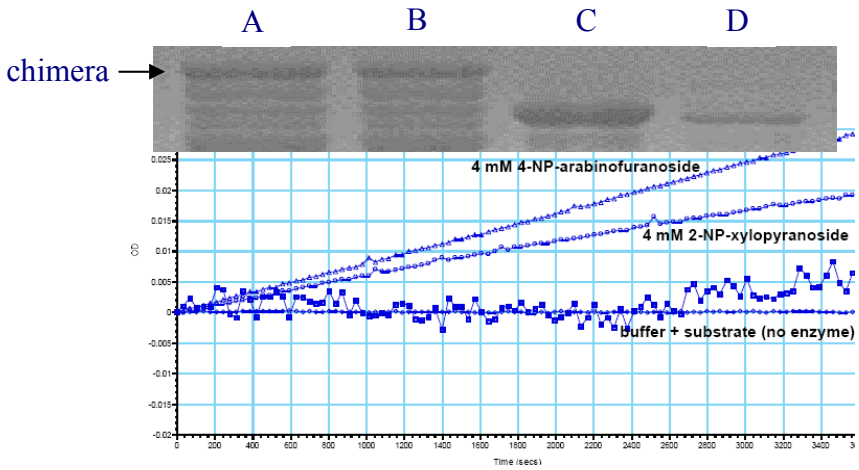
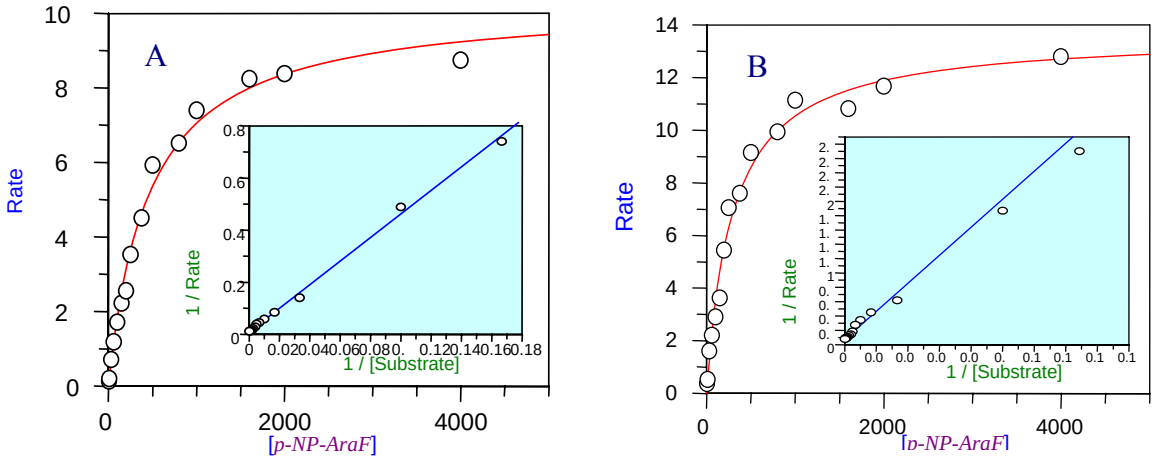


Figure 5. Recombinant enzyme production and activity assay of the arabinofuranosidase-xylosidase chimera. Upper panel: A, total lysate of *E. coli* expressing the fusion gene. B, soluble fraction of the fusion gene-expressing *E. coli*. C, soluble fraction of the arabinofuranosidase-expressing *E. coli*. D, soluble fraction of the xylosidase-expressing *E. coli*. The fusion protein was purified and assayed using substrates indicated in the graph.

We characterized the chimera's biochemical properties and kinetics and compared them to those of the individual parental enzymes. Under similar induction conditions for the parental genes,

soluble, His-tagged recombinant chimera proteins can be produced from *E. coli* cells and purified using Ni-NT columns. Under identical buffer and assay conditions, the chimera has comparable K_m, k_{cat} and k_{cat}/K_m values as the parental enzymes (Fig. 6). Furthermore, when p-NP-araF was used as substrate, the chimera has the same pH optimum as the parental arabinofuranosidase (pH 6.0; Fig. 7). Overall, the chimera has similar biochemical properties and kinetics to the parental enzymes.



Enzyme	Substrate	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1}sec^{-1}$)
xylosidase	p-NP-xylP	93 ± 10	3.8 ± 0.2	41
arabinofuranosidase	p-NP-araF	398 ± 34	6.2 ± 0.2	15.6
Chimera	p-NP-xylP	20 ± 4	0.94 ± 0.04	47
	p-NP-araF	366 ± 19	9.6 ± 0.2	26.3

Figure 6. Kinetic analysis of theparental and chimeric enzymes. A and B: initial velocities of the single araF and chimera, respectively; the inserts are reciprocal plots of the initial velocities. The lower table summarized the kinetic parameters of the single enzymes and chimera. Results were obtained at pH 6.0 at 35°C.

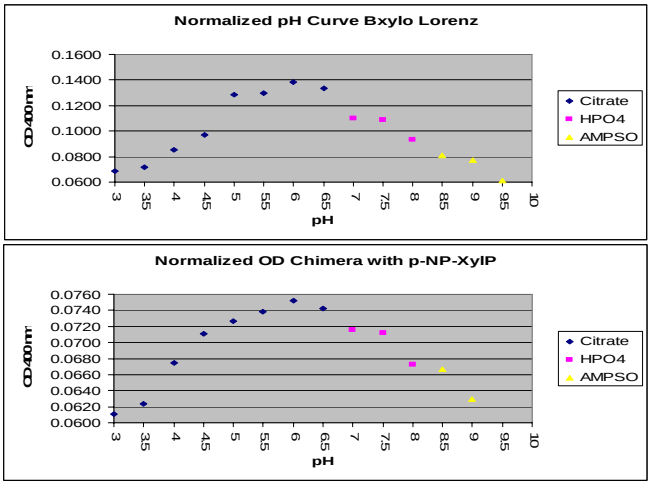


Figure 7. pH optima of AraF (upper panel) and chimera (lower panel) using p-NP-AraF as substrate. Citrate buffer (pH 3 – 6.5), HPO4 buffer (pH 7 - 8) and AMPSO buffer (pH 8.5 – 9.5) were used. Assays were performed at 35^{o}C.

Creation of a Xylanase-xylosidase-arabinofuranosdase fusion enzyme. To generate a trimeric hydrolase, we have linked a *Clostridum* xylanase (see below for description) to the N-terminus of the above-described chimera. The fusion gene in a pET29a vector was expressed in *E.coli* cells and the colonies were assayed for xylosidase activity using the direct colony assay described above. The result indicates that the trimeric enzyme has xylosidase activity. The *E.coli* cells were grown in liquid culture, and soluble proteins were isolated from cell lysates and used for xylanase activity assay. Our results indicate that the trimeric enzyme is soluble and enzymatically active. We are currently performing biochemical and kinetic characterizations of the trimeric enzyme similar to those described above for the chimeric enzyme.

5. Expression and purification of single and polymeric enzymes

The *Clostridium* xylanase, the xylosidase-arabinofuranosidase chimera and the trimeric xylanase- xylosidase-arabinofuranosidase were expressed in *E. coli* and purified using Ni-columns.

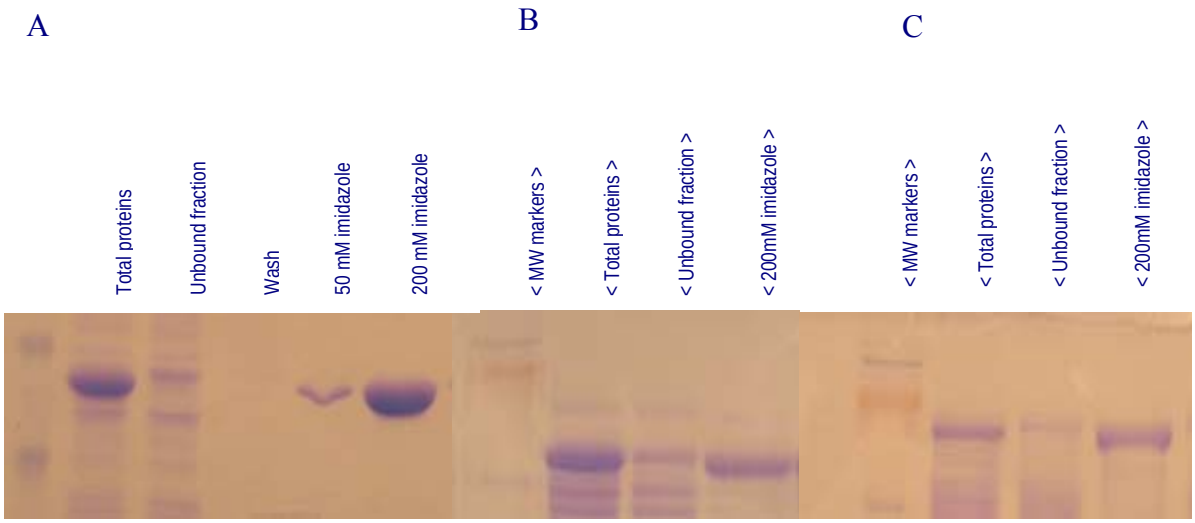


Figure 8. SDS-PAGE analysis of purified recombinant xylanase, chimeric arabinofuranosidase-xylosidase and trimeric xylanase- arabinofuranosidase-xylosidase.

As shown in Figure 8A, the His-tagged xylanase is produced as more than 70% of the total soluble *E. coli* proteins and can be purified using a Ni column with a gradient of imidazole. The high-level expression of the xylanase in both transgenic plant (see proposal) and *E. coli* reaffirms our proposed strategy of using this protein as an anchor for polymeric enzyme production. High-levels of soluble chimeric (Fig. 8B) and trimeric enzyme (Fig. 8C) are also produced and the enzymes can be purified using similar procedures for the purification of xylanase.

Kinetic Analysis of fusion proteins. In our effort to generate fusion enzymes for lignocellulose degradation, the xylosidase-arabinofuranosidase chimera and the trimeric xylanase-xylosidase-arabinofuranosidase have been expressed at high-levels in *E. coli* and purified for characterization. Kinetic analysis of the chimera enzyme shows that the dual functional enzyme has similar enzymatic specificities as the individual parental enzymes (Table

2). The chimera also has similar pH and temperature optima as the parental enzymes. A multi-functional fusion enzyme can be further improved as a single protein using directed evolution.

Enzyme	Substrate	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1}sec^{-1}$)
xylosidase	p-NP-xylP	93 ± 10	3.8 ± 0.2	41
arabinofuranosidase	p-NP-araF	398 ± 34	6.2 ± 0.2	15.6
Chimera	p-NP-xylP	20 ± 4	0.94 ± 0.04	47
	p-NP-araF	366 ± 19	9.6 ± 0.2	26.3

Table 2. The kinetic parameters of the single enzymes and chimera. Results were obtained at pH 6.0 at 35°C.

6. Transformation of the trimeric gene to tobacco

The gene encoding the xylanase-xylosidase-arabinofuranosidase was cloned into the binary vector under the control of the MMV promoter and introduced into tobacco via *Agrobacterium*-mediated transformation as described previously (Pattanaik, S. et al. 2004. Plant Sci 167:427). Two constructs have been used to express the trimeric enzyme either cytoplasmically or in apoplasts through linkage of a targeting signal peptide. Ten to twenty independent lines for each construct are currently being grown in greenhouse.

7. New chimeric and trimeric enzymes using different peptide linkers

To investigate whether or not different peptide linkers can affect function of the polymeric enzymes, we constructed new xylosidase-arabinofuranosidase and xylanase-xylosidase-arabinofuranosdase in which the 3 or 6 poly-glycine linkers were replaced with one of three linkers: (KLAIGPMYNQVVYQYPN), (KLGGGGGGGGG) and (GGGGGADP). These linkers have been shown to be effective in other fusion proteins. The new chimeric and trimeric enzymes have been produced in *E. coli* and purified as described above and will be characterized

kinetically. The enzymatic comparison will allow us to select appreciate linkers for optimal enzyme activity.

8. Construction of a tetrameric enzyme and new trimeric enzymes

To test the feasibility of creating a functional tetrameric enzyme, we linked an α-glucuronidase or an acetylxylan esterase, recently cloned by Charles Lee of USDA, to the C-terminus of the trimeric enzyme. We have also created a new trimeric xylanase-glucuronidase-acetylxylanesterase. SDS-PAGE has confirmed the productions of these proteins. We are currently characterizing these two new polymeric enzymes using enzyme activity assays and kinetic analysis. Genes encoding additional cellulosic hydrolases are being used to generate new polymeric enzymes.

9. Development of a capillary electrophoresis method for monitoring natural substrate hydrolysis

Cereal grain natural arabinoxylan substrates have homopolymeric backbones of (1-4)-β-D-xylopyranosyl residues substituted with (1-2)- and/or (1-3)-α-linked L-arabinofuranosyl branch units. The wheat arabinan medium viscosity arabinoxylan tested (Megazyme) has an arabinose:xylose:other sugars ratio of 37:61:2. Capillary electrophoresis (CE) was used to monitor the degradation of this natural substrate by the single active site parental arabinofuranosidase and xylosidase, both alone and in combination. These results will be compared to incubation of the natural substrate with the chimeric (dual active site) enzyme to detect potential synergetic effects. The amounts of the monomeric parental arabinofuranosidase

and xylosidase tested were adjusted to match the specific activity of the chimera with respect to activity on p-NP-xylopyranoside and p-NP-arabinofuranoside.

Enzyme samples were incubated with the natural substrates. Unreacted substrate was precipitated by adding EtOH and cooling at –20 °C. The samples were centrifuged, and an aliquot from each reaction was lyophilized prior to derivatization. The 1-Aminopyrene-3,6,8-trisulphonate (APTS) derivatization was performed and allowed to occur overnight at 37 °C. The samples were then stored at -20 °C and aliquots diluted 200 to 400-fold prior to capillary electrophoresis. Analyses were performed on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Inc., Fullerton, CA). Separations were performed using a 20 cm uncoated fused-silica capillary column of 50 μm internal diameter (MicroSolv Technology Corp., Long Branch, NJ). The detection system was a Beckman laser-induced fluorescence detector using an excitation wavelength of 488 nm and detection at 520 nm.

10. Deliverables

We submitted abstracts and registered to attend two conferences on bioenergy research in 2007: the 29th Symposium on Biotechnology for Fuels and Chemicals, April 29th, Denver, CO, and NABC 19: Agricultural biofuels, technology, sustainability, and profitability, May 22nd, Brookings, SD. A grant proposal was submitted to USDA based on the work partially supported by GOEP on directed evolution of the hemicellulosic hydrolases and received funding for 2006-08.

Appendix A: Reporting Requirements

Project Schedule and fulfillment

Task	Resource	Project Month											
		1	2	3	4	5	6	7	8	9	10	11	12
Express xylosidase and arabinofuranosidase in *E. coli*	Z. Fan	**X**	**X**	**X**	**X**								
Express xylosidase and arabinofurnaosidase in *Pichia*	Z. Fan												
Construct enzyme libraries	Z. Fan		**X**	**X**	**X**	**X**	**X**						
Construct xylosidase-arabinofuranosdiase fusion protein	Z. Fan			**X**	**X**	**X**	**X**	**X**	**X**				
Construct fusion enzyme library	Z. Fan												
Develop arabinofuranosidase assay using 5-BIara	K. Wagschal	**X**	**X**	**X**	**X**	**X**	**X**						
Screen arabinofuranosidase library for xylosidase activity	Z. Fan					**X**	**X**	**X**	**X**	**X**			
Screen xylosidase libraries for activity at 30°C	Z. Fan												
Screen fusion enzyme libraries for increased activities	Z. Fan												
Kinetic analysis of selected enzyme variants	K. Wagschal					**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
Test improved enzyme using corn fiber	K. Wagschal										**X**	**X**	**X**
***Characterize additional xylosidase and arabinofuranosidase**	**K. Wagschal**	**X**	**X**	**X**	**X**	**X**	**X**	**X**					
***Construct trimeric enzymes**	**Z. Fan**					**X**	**X**	**X**	**X**	**X**	**X**	**X**	
***Develop assays using capillary electrophoresis with natural substrates**	**K. Wagschal**					**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
***Transform fusion enzyme genes into transgenic plants**	**Z. Fan**							**X**	**X**	**X**	**X**	**X**	**X**
Progress Report #1	L. Yuan												
Draft Final Report	L. Yuan												**X**

* New tasks not included in the proposal.

Appendix B: Proposal

Directed Evolution of Enzymes for the Conversion of Corn-Fiber for Biofuel Production

Specific Goals: (1) Use directed evolution technologies to improve the enzyme specificities and temperature optima of □-xylosidase and arabinofuranosidase, key enzymes for corn fiber degradation. **(2)** Create a single enzyme with dual arabinofuranosidase/xylosidase activities in order to reduce the number of enzymes required for biomass conversion. **(3)** Generate preliminary data for the application of additional full grants from federal agencies.

Introduction

Kentucky's comprehensive energy strategy calls for the enhancement of the production, consumption and availability of renewable energy resources within the state. The most abundant sources of renewable carbon in nature are plant structural polysaccharides. One of the major goals of the biorefinery industry is to maximize the use of biomass feed stocks by producing a wide range of products including fuels. Ethanol made from corn is already used as an additive in about 10% of the gasoline sold in the United States. The added ethanol boosts octane and reduces carbon monoxide emissions. The use of biofuels also reduces the dependence on petroleum. Currently, US ethanol fuel is mainly derived from grain (mostly corn) starch. The starch content is only about 71% of the corn kernel. The protein/fat and leftover fibrous materials (30-50% arabinoxylan, 15-20% cellulose and 10-25% residual starch), referred to as corn fiber, are processed into animal feed. There is considerable interest in the exploitation of corn fiber for the generation of biofuels. The National Renewable Energy Laboratory (NREL) estimates that utilization of corn fiber can increase corn-based ethanol production by 13%, from

46 L/100 kg corn to 52 L/100 kg corn (Technology Brief, NREL, 11/1993). The conversion of corn fiber to fermentable sugars adds value to the corn milling process as the protein/fat fractions can still be utilized as animal feed supplement. In order to convert the residual starch and hemicellulosic polymers in the relatively insoluble corn fiber, the biomass needs to be hydrolyzed. Chemical hydrolysis of corn fiber is efficient but generates waste and fermentation inhibitors. Enzymatic saccharification is desirable as a biocompatible, environmentally responsible alternative. Moreover, it should be noted that enzymatic saccharification of starch has supplanted chemical hydrolysis due to an economic driving force, and it is anticipated that a similar scenario will play out for hemicellulose saccharification. However, complete enzyme hydrolysis of cellulosic biomass is not yet cost-effective, and the currently available commercial hydrolases have shown poor results in corn fiber saccharification. A mixture of five commercial enzymes only resulted in partial saccharification of the chemically pretreated corn fiber (Hespell, RB et al. 1997). The major component of corn fiber, xylan, is a complex heteropolysaccharide with a β-1,4-xylose backbone rich in arabinose side chains. Endo-xylanase is the primary enzyme that attacks the backbone structure by randomly cleaving the internal xylosidic linkage, and the products are further hydrolyzed by β-xylosidase (exo-xylanase). However, the complex side-chain structures (mostly arabinose, together with glucuronic acid/ester and acetic, ferulic and coumeric acids) greatly reduce the accessibility of the backbone. Thus saccharification of corn fiber may require additional enzymes, including β-xylosidase, α-L-arabinofuranosidase, α-glucanronidase, acetyl xylan esterase and feruloyl esterase (Saha and Bothast, 1999). Therefore, the challenges facing the cost-effective, sufficient enzymatic saccarification of corn fiber include (1) significant increase of activities of the enzymes required for the process, (2) reduction of the

number of enzymes required for the process and (3) large scale, low cost production of the enzymes.

We propose to address these challenges by the application of directed protein evolution technologies. Directed evolution is a general term used to describe various techniques for the generation of mutants (variants) and selection of desired functions. Darwin described the evolution of life as a competitive process of "variation and natural selection" arising from successive cycles of mutation and adaptation. Directed evolution incorporates Darwinian principles of mutation and selection into experimental strategies for improving biocatalyst or cellular properties. In the laboratory, directed evolution comprises two discrete components: first, genetic diversity is created through the production of a library of genetic variants, and second, the library is evaluated through genetic selection and high-throughput screens to identify variants with the required function(s). Point mutation and recombination are the two primary processes for the generation of genetic diversity. Iteration of a directed evolution cycle facilitates the accumulation of beneficial mutations while eliminating deleterious ones (Figure 1) (Yuan L. et al. 2005; Chatterjee and Yuan, 2005). Directed evolution differs from natural evolution in two key aspects: i) natural evolution occurs under multiple and variable selection pressures, whereas directed evolution is performed under controlled selection pressure for predetermined functions and ii) in directed evolution, "non-natural" functions of practical use may be derived through the design of appropriate selection schemes, while natural evolution is primarily guided by functions advantageous to the survival of the organism.

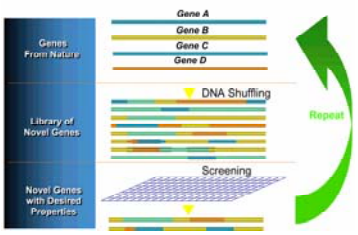


Figure 1. DNA shuffling as an example of directed evolution. A set of homologous parental genes or variants of a single gene differing by point mutations are fragmented and subsequently reassembled. The resulting library of gene variants is screened according to defined parameters to isolate progeny with desirable properties. The selected progeny are then used as parents for subsequent rounds of breeding (Stemmer, 1994).

In this study we are interested in evolving the genes encoding two classes of enzymes, xylosidase and arabinofuranosidase, that have been characterized in our USDA collaborators' laboratory. These classes comprise some of the synergetic enzymes for saccharification of corn fiber that require further optimalization (Sørensen HR et al. 2005). For example, the xylosidase from *Thermoanaerobacterium* sp. strain JW/SL YS485, is thermostable up to 70^{o}C, but has an activity maximum at 65^{o}C (Lorenz and Wiegel, 1997). It is thus desirable to change the activity maximum to lower temperature, allowing the saccharification to occur at lower temperature which leads to a significant saving in energy. In addition, it is also desirable to engineer the arabinofuranosidase to possess xylosidase activity. Using an enzyme with dual activities reduces the cost of production for an additional industrial enzyme, another major saving in cost and energy.

We propose to engineer the (glycoside hydrolase) family 51 arabinofuranosidase from *Geobacillus sterothermophilus*, and family 39 xylosidase from *Thermoanaerobacterium*, to demonstrate the feasibility of using directed evolution to create a hydrolase with dual function. The 3-dimensional structure of this arabinofuranosidase shares high similarity with that of the family 39 β-xylosidase (Hovel et al. 2003; Yang et al. 2004). Both the 51 and 39 enzyme families belong to the $(\beta\alpha)_8$-barrel superfamily, a versatile protein family with an ancestral scaffold that represents a target well-suited for novel protein design through directed evolution

(Hocker B. 2005). Although the family 51 and 39 enzymes share no sequence homology, the 8 active-site residues between the two enzymes can be superimposed. In addition to the hydrolyzing activity on its native substrate, the family 51 arabinofuranosidase also has weak activity on synthetic substrates such as 4-methylumbelliferyl and aryl-β-D-xylopyranosides which have good leaving groups (Whitehead and Cotta, 2001; Hovel et al. 2003). These data indicate that it may be possible to evolve xylosidase activity on native (natural) substrates into this enzyme. Directed evolution technologies are a powerful tool to introduce these "non-natural" but highly desirable functions into enzymes. The available 3-dimensional structures of the two enzymes can guide the creation of more targeted sequence diversity and assist in the functional interpretation of mutations in the evolved variants. More importantly, our proposed approaches may prove applicable to other enzymes that are involved in biomass conversion.

Research Description

Project Objectives:

(1) Improvement of enzymatic activity of the *Thermoanaerobacterium* β-xylosidase at 30^{o}C.

(2) Evolution of the family 51 arabinofuranosidase gene to encode an arabinofuranosidase/xylosidase with dual specificity on natural substrates.

(3) Alternatively, creation and evolution of an arabinofuranosidase/xylosidase fusion protein for increased activities on corresponding natural substrates.

(4) Development of assays for the characterization of corn fiber hydrolysis by the evolved enzymes.

Past Progress and Current Pertinent Studies:

(1) Cloning and recombinant expression of hemicellulose degradation enzymes. Our partners in this project, the USDA scientists at the Western Regional Research Center, Albany, CA, have

been pursuing gene discovery and improvement of enzymes that degrade biomass. The USDA Center has an extensive collection of gene sources for biomass hydrolytic enzymes including those being used in this study. The USDA collaborators are also the leading experts in recombinant expression, analysis and structure/function characterization of these enzymes (Lee CC. et al 2001; Lee CC et al. in press; Wong DWS 2003; Wagschal K et al. 2005).

(2) Development of high-throughput assays for xylosidase and arabinofuranosidase. Our USDA collaborators have recently developed a xylosidase assay using natural substrates (Wagschal K et al. 2005). This enzyme-coupled assay monitors the production of intermediary hydrogen peroxide and can be adopted for both liquid and solid-phase formats (see Screening Enzyme Library section below). The assay is particularly useful in high throughput enzyme library screening because it can be applied to various direct cell-based screenings including colony lift and high-density grids in 96- or 384-well formats, and because the enzyme activity can be detected and quantified by multiple means including spectrophotometry and image pixel densitometry. Another advantage of this assay system is that it utilizes natural xylooligosaccharides instead of artificial substrates. An efficient screening system for the arabinofuranosidase has also been developed (see Screening Enzyme Library section below).

(3) Directed evolution of temperature and pH optima. The PI has been involved in a collaborative project with Pioneer HiBred which resulted in the successful modification of the thermostability and pH optimum of an amine oxidase, an enzyme involved in detoxification of the mycotoxin fumonisin. Libraries of amine oxidase were expressed in *E. coli* and *Pichia pastoris,* and screened at elevated temperatures and/or pH 5.5 (the wild-type enzyme has a pH

optimum of 8 that is not ideal for functioning in extracellular apoplasts where the pH is 5.5). Significantly improved variants that retain full activity at up to 40^{o}C were obtained. One variant exhibited not only a 20-fold increase in k_{cat}/K_m, but also a shift in pH optimum from 8 to 5.5 (Chatterjee R et al. manuscript submitted; Yuan et al. 2005).

(4) Directed evolution of dual enzymatic activities. The amine oxidase has been evolved to catalyze its non-natural substrate, fumonisin B1, for the purpose of reducing the detoxification pathway from two enzymes to one. After screening a large number of variants using fumonisin B1 as substrate, a number of variants have been identified to have an increased k_{cat}/K_m that is comparable to that of the wild-type enzyme for its natural substrate (Chatterjee et al. manuscript submitted; Yuan et al. 2005).

(5) Creation of fusion proteins. A bacterial carotenoid biosynthetic gene (*crtI*) and a plant phytoene synthase (*psy*) have been used to create a fusion protein (crtI-psy), linked by a poly-glycine peptide, for production of the antioxidant □-carotene. The fusion protein has dual function and is capable of catalyzing the two respective substrates, GGPP and phytoene, to produce carotene. The fusion protein has been expressed in *E. coli* and subjected to DNA shuffling. The library of variants was visually screened based on color density and resulting variants with increased carotene production were identified. Transgenic rice expressing the evolved fusion crtI-psy protein accumulated significantly higher amounts of □-carotene than those co-expressing the two wild-type enzymes (L. Liu, unpublished results; Yuan, L. et al. 2005)

Experimental Plans:

(1) Expression of xylosidase and arabinofuranosidase in *E. coli* and *Pichia*. Both xylosidase and arabinofurnaosidase can be readily expressed in *E. coli* using the pET vectors (Wagschal, et al. 2005; Beylot MH et al. 2001). The *E. coli* cells expressing the enzymes will be used in high throughput screening using the methods described by Wagschal et al. (2005). On the other hand, the *Pichia pastoris* recombinant gene expression system is an excellent tool for production and selection of glycoside hydrolases (Berrin JG et al. 2000). In the *Pichia* system the recombinant enzymes are secreted to the outside of the cells and thus allows direct detection of enzyme activity on colonies. This will be particularly useful in selection of the primary library where a mixture of active and inactive clones exists. Active clones will first be selected for re-array and screened for improved or modified specificity using a second substrate. For expression of xylosidase and arabinofuranosidase in *Pichia*, the coding gene of each enzyme will be cloned into the pPICZ vector (Invitrogen) in which the *AOX1* promoter controlled expression can be induced by methanol. *Pichia* transformants will be re-arrayed onto a second plate and assayed for activities under a variety of conditions (see below). One potential problem with the *Pichia* system could be the presence of native glycoside hydrolase activities that may interfere with the recombinant enzyme activities. However, based on our past experience this appears unlikely due to the high-level expression of the recombinant enzymes. In the instance that high background activity is encountered, the *E. coli* expression system will be used.

(2) Generation of enzyme libraries. The enzyme variant libraries will be constructed using a number of techniques including error-prone PCR, single-gene shuffling, family-gene shuffling and synthetic-oligo spiking (Yuan, L et al. 2005). Error-prone PCR has been successfully applied to evolve a variety of polysaccharide hydrolases, e.g. □-amylase (Wong, DW et al.

2004). In the single gene shuffling process, the hydrolase genes will be randomly fragmented by DNase I, and the resulting fragments are reassembled based on sequence similarity by primerless PCR. Mutations are introduced according to the error rate of the polymerase. The correct DNA fragments are then rescued by PCR with appropriate forward and reverse primers. When this process is repeated, new combinations of mutations are formed within a conserved sequence framework and additional point mutations may also arise. In contrast to single-gene shuffling where library members are typically 95-99% identical, family-shuffling allows block exchanges of sequences that are typically >60% identical. Larger numbers of mutations are tolerated within a given sequence without the introduction of deleterious effects on structure and/or function partly because sequence diversity derives from related, parental sequences that have survived natural selection ("functional" sequence diversity). We will take advantage of the genetic diversity collection of the glycoside hydrolases in our USDA collaborators' laboratory and will introduce multiple xylosidases or arabinofuranosidases in the library construction.

In addition to evolving arabinofuranosidase gene to encode an enzyme with dual-specificities, a fusion protein can be developed by fusing the two enzymes with a peptide linker, similar to what has been achieved with the fusion protein, crtI-psy (see above). A variety of peptide linkers, including poly-glycine or peptide TGEKP or GEKKF, will be tested for optimal function. Once the functional fusion xylosidase/arabinofuranosidase is obtained it will be used as a single enzyme in directed evolution for further improvement of both enzyme activities.

(3) Screening of the enzyme libraries. The solution phase assay for β-xylosidase natural substrate hydrolysis activity will be performed in two steps. The first step is an endpoint assay of

the β-xylosidase enzyme, where the glycosidic bonds of a soluble xylooligosaccharide substrate (xylobiose or xylotriose in this study) are hydrolyzed by the enzyme, and D-xylose is generated. In the second step, the amount of xylose liberated is quantified using an enzyme-coupled kinetic assay in which pyranose oxidase converts the D-xylose hydrolysis product to 2-keto-D-xylose with the concomitant formation of H_2O_2 (Equation 1). In a subsequent reaction catalyzed by horseradish peroxidase, the hydrogen peroxide generated oxidizes the chromagen ABTS (azino-bis-ethylbenzothiazoline-sulfonate) to form the colored ABTS radical cation (Equation 2), with the rate of formation being monitored at 420 nm. The D-xylose concentration in the assays can be calculated using a D-xylose standard curve generated on the same microtiter plate.

(1) $$D\text{-}xylose + O_2 \xrightarrow{pyranose\ oxidase} 2\text{-}keto\text{-}D\text{-}xylose + H_2O_2$$

(2) $$H_2O_2 + 2ABTS(H) \xrightarrow{peroxidase} 2H_2O + 2ABTS^{\bullet+}$$

A solid-phase variation (useful for primary screening) of the assay has also been developed that uses the chromagen 3,3'-diaminobenzidine tetrahydrochloride (DAB), which in the presence of H_2O_2 in a peroxidase catalyzed reaction, produces an insoluble brown precipitate.

For detection of the arabinofuranosidase activity, solution phase kinetic spectrophotometric assays will be performed using the substrate *p*-nitrophenylα-L-arabinofuranoside and measuring the change in absorbance at 400 nm. Rates of hydrolysis are obtained from initial slopes. The rate of *p*-nitrophenyl α-L-arabinofuranoside hydrolysis will be quantified using standard curves generated with *p*-nitrophenol. A solid-phase variation of the assay will be carried out using the substrate 5-bromo-3-indoyl-α-L-arabinofuranoside (5-BIara) (Berlin, W and Sauer, B. 1996); hydrolysis of which releases the blue-colored chromophore 5-bromo-3-indolyl. Colonies

expressing arabinofuranosidase activity can be visualized (blue color) by incubation in LB medium containing 5-BIara. The advantage of this assay is that it is highly sensitive due to low background interference. The substrate 5-BIara will be synthesized by SRI Biosciences, Inc., Mountain View, CA.

The xylosidase library in *E. coli* will be robotically gridded from master plates using a Q-Pix colony picker onto a Nytran SPC membrane. The membrane will then be placed onto LB agar containing IPTG. Following overnight incubation at 30^{o}C, the membrane will be carefully lifted from the growth/induction agar and placed face-down onto a low-melting agar plate containing lysis buffer, xylooligosaccharides, pyranose oxidase and peroxidase according to procedures described by Wagschal et al. (2005). Lysis and hydrolysis of the mixed xylooligosaccharides will be allowed to occur under specified selection conditions, and the membrane will be analyzed using an AlphaImager imaging system. The enzyme library in *Pichia* will be screened using a similar protocol except the gene expression will be induced by methanol and cells will not be lysed. For screening of xylosidase variants with improved activity at 30^{o}C, the libraries will be assayed at 30^{o}C and colonies showing higher activities (higher color intensity) will be selected and re-assayed using the more quantitative liquid assay. The mutant genes will be combined as parents for the subsequent round of evolution. For screening of the dual-specificity xylosidase/arabinofuranosidase, the individual libraries of family 51 arabinofuranosidase will be first assayed for retention of activity on 5-BIara. Then the active clones will be re-arrayed and assayed for the novel activity on the natural substrate xylobiose. Clones with detectable novel activity will be combined as parents for the subsequent round of evolution.

(4) Characterization of the improved variants. Selected variants with improved enzyme specificities will be characterized for their activities on natural corn fiber with or without additional hemicellulolytic enzymes using procedures described by Koukeikolo et al. (2005). Sequence analysis of these mutants will be used to identify potential "hot spots" - regions or domains critical for enzyme specificity. If potential hot spots can be identified, targeted site-directed mutagenesis, saturation mutagenesis and/or cassette mutagenesis will be performed to create localized sequence diversity for selection of further improvements. We have successfully applied this approach to evolve the transcriptional activity of a basic loop-helix-loop type transcription factor (Pattenaik et al. submitted). Mutations in improved variants will be interpreted based on the 3-dimensional structures of the xylosidase and arabinofuranosidase. Information thus generated will be used to guide our continuing efforts in the engineering of novel activities for enzymes involved in biomass conversion.

Significance and Feasibility

We will address one important aspect of the State of Kentucky's commitment to technology development, production and utilization of renewable energy. Biofuels derived from cellulose/hemicellulose have the advantages of high-level sustainability, obtaining from non-food portions of renewable feedstocks and potential to have large-scale impact to our state's and national agriculture. Corn fiber is a byproduct of the existing corn milling process thus an excellent value-added target for production of biofuels. The technology for making ethanol from corn fiber was honored by *R&D* magazine as one of the 100 most important technological innovations of the 1990s.

The xylosidase and arabinofuranosidase are critical enzymes in enzymatic saccharification of corn fiber. Although not the only enzymes needed in the process, xylosidase and arabinofuranosidase play important and synergetic roles in hemicellulose degradation. The ability to optimize the enzyme activities at a milder temperature can lead to reduction of energy for the bioconversion. Development of an enzyme with dual-specificity will not only provide insight into protein structure/function relationships, but will also result in significant cost and energy savings for the production of the hydrolases. More importantly the approaches, if successful, will be applicable to other enzymes involved in the process such as xylanase and □-glucanronidase.

This project is technically feasible. The PI's group and the USDA collaborators are experienced in all aspects of molecular biology, directed protein evolution and high throughput screening required for this proposal. We have multiple gene sources for the targeted enzymes. The PI has been involved in directed evolution research for almost ten years and is the corresponding author of two very recent articles on this topic (see CV). The USDA scientists are leading experts in polysaccharide hydrolases and have published numerous papers on the identification, characterization and modification of these enzymes. All techniques and equipment needed for the proposed project (including those to be used for high throughput liquid handling and high density cell array) are available in either the PI's or the USDA laboratory. This seed grant will support the first phase of our research and allow us to generate sufficient data for applications of additional research grants for the next phase that includes broadened enzyme targets and more in-depth understanding, design and engineering of enzymes for biomass conversion.

This project emphasizes and stimulates interactions and collaborations between the PI and the USDA scientists. The breadth of interests of the collaborative team provides expertise and experiences in bioinformatics, genetics, protein engineering, enzyme screening and biochemistry, and bioprocessing. This is critical for successful implementation of the experimental approaches and interpretation of the results. Our long-term goals include expansion of the collaboration in research as well as conversion of the research results into practical applications that directly benefit US farmers and businesses.

Timetable

Tasks	Q 1	Q 2	Q 3	Q 4	Laboratory
Express xylosidase and arabinofuranosides in *E. coli*	→				USDA
Express xylosidase and arabinofuranosides in *Pichia*	→	→	→	→	UK
Construct enzyme libraries					UK
Construct xylosidase-arabinofuransidase fusion protein		→	→		UK
Construct fusion enzyme library			→	→	UK
Develop arabinofuranosidase assay using 5-BIara	→	→			USDA/UK
Screen xylosidase libraries for activity at 30^{o}C		→	→		UK
Screen libraries arabinofuranosidase for xylosidase activity		→	→	→	UK
Screen fusion enzyme libraries for increased activities			→	→	UK
Kinetic analysis of selected enzyme variants				→	USDA
Test improved enzymes using corn fiber				→	USDA

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Directed Evolution of Enzymes for the Conversion of Corn-Fiber for Biofuel Production

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Key Challenges Facing Biomass Conversion

- Improving efficiency
- Lowering costs

Some Practical Solutions:

- Simplified processes, e.g. SSC
- Reducing number of enzymes needed*
- Plants as enzyme factories**

*in collaboration with Drs. K. Wagschal and C. Lee, USDA/ARS/WRRS
**in collaboration with Drs. S. Nokes, M. Montross and I. Maiti, UK

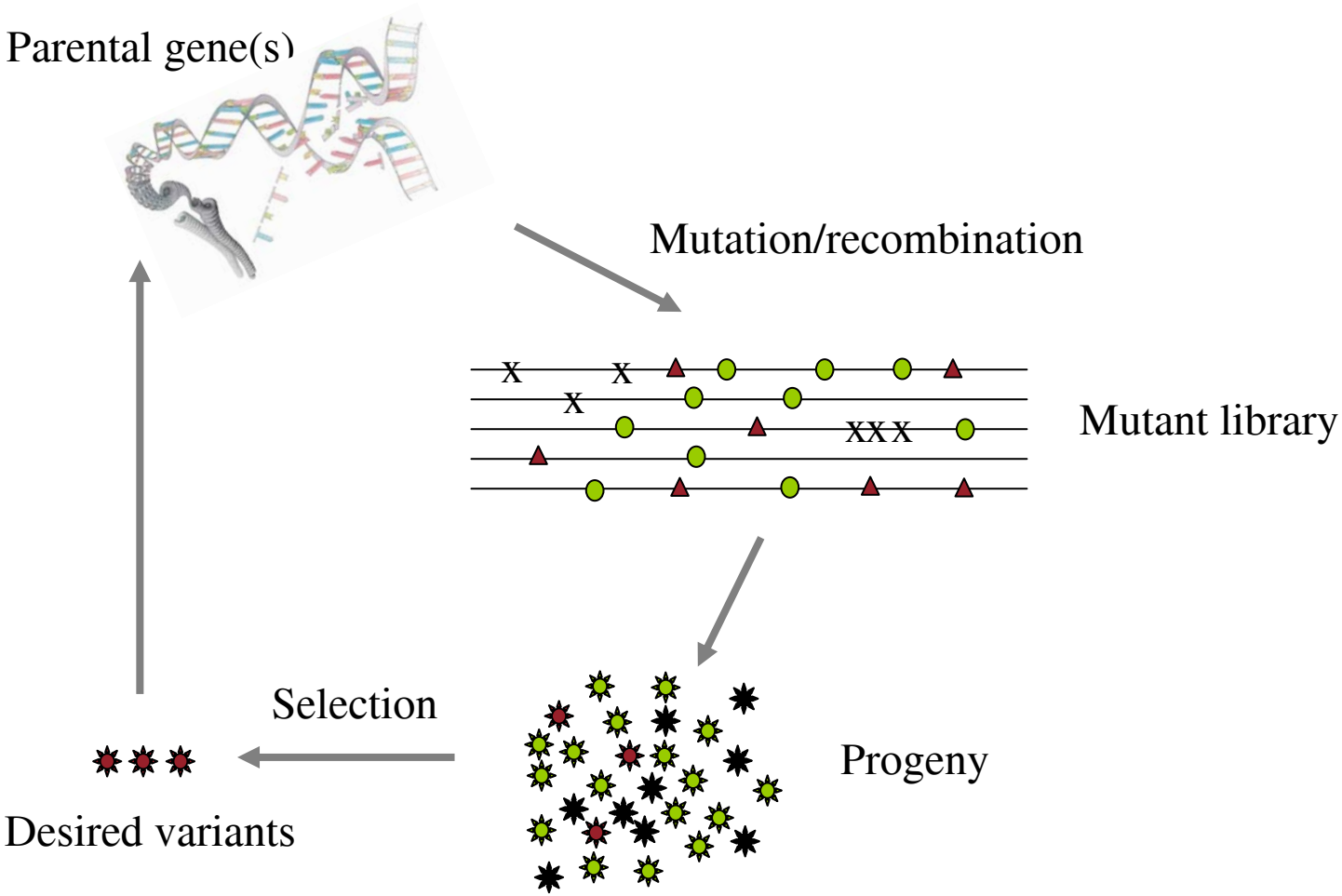
Our Approach to Reducing Number of Enzymes

Think of it as the minimum number of utensils
you need to take to a picnic…

Instead of spoons, forks, knives, cork-opener and scissors,
you only need two…

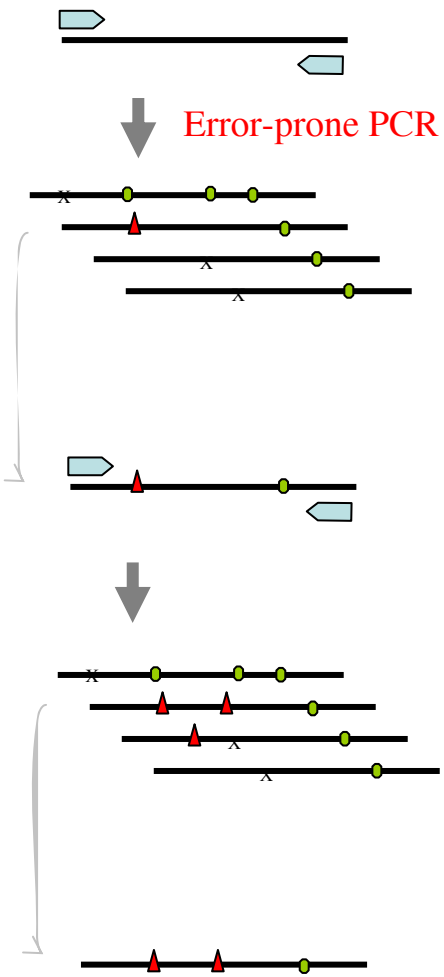
"The **spork** is the only true American utensil" John Nihoff,
gastronomy professor at the Culinary Institute of America

How To Make Sporks: Directed Protein Evolution

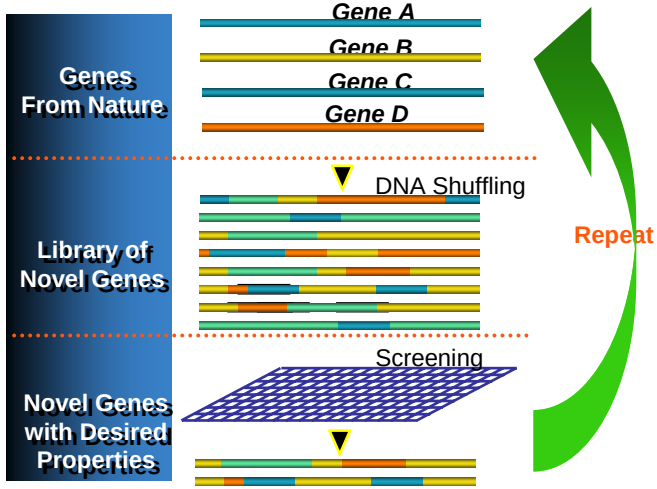


Directed Evolution Comes with Multiple Formats

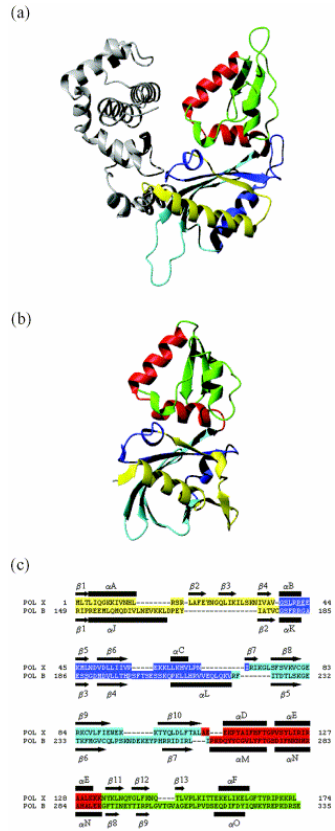
Asexual

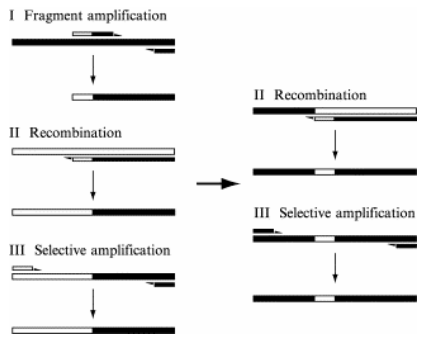


Sexual



Structural





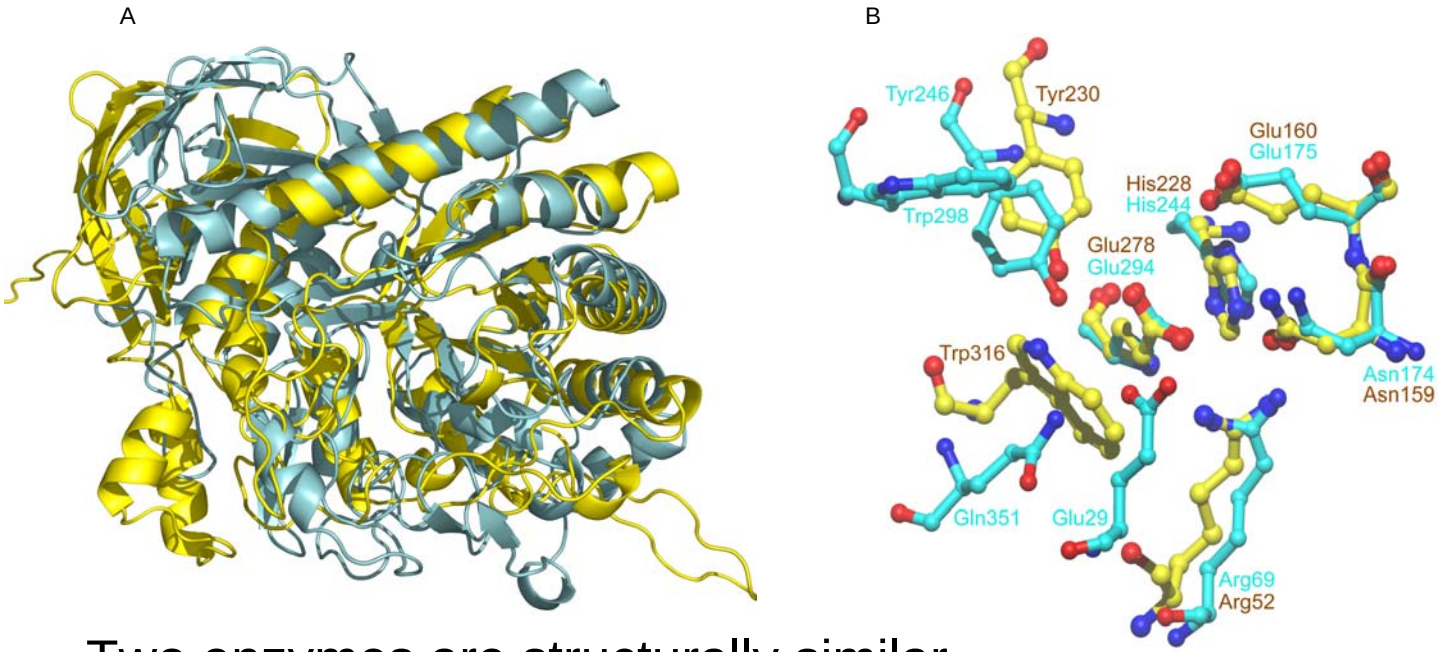
Accelerated Evolution

Millions years

< One year

The First Spork: a bi-functional hemicellulase

- (family 39) Xylosidase (*Thermoanaerobacterium*)
- (family 51) Arabinofuranosidase (*Geobacillus*)



- Two enzymes are structurally similar
- No significant cross substrate specificity
- Ideal target for directed evolution
- Spork is risky

Swiss Army Knife Approach: fusion enzymes

- Fusion protein: two or more proteins fused by a linker
- Expressed and produced as a single protein
- Can be further improved by directed evolution

- Advantageous when expressed in plants
    - Reduce the number transgenes
    - In some cases may improve production

Plants As Factories for Biomass-Degradation Enzymes

- As substitution of fermentation-based industrial enzymes
- For self-degradation of biomass

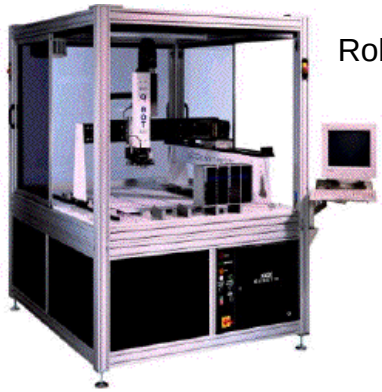
Challenges:

- Difficult to produce large numbers of enzymes in plants
    - difficulty of gene transformation
    - difficulty of downstream breeding

A possible solution:

- Use Sporks and Swiss-Army-Knives
    - possible to put 3-4 single genes in one construct
        = 6-8 enzymes

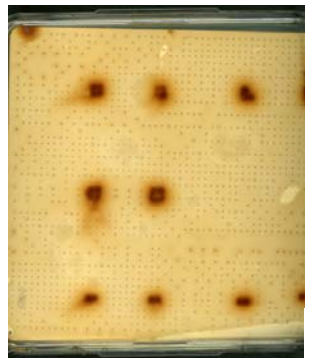
We Are Making Progress



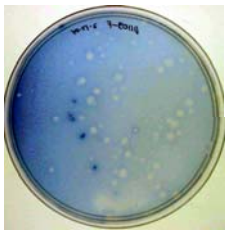
Robotic rearray

Liquid handling

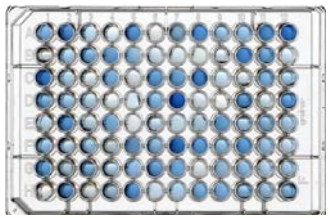
HD array assay



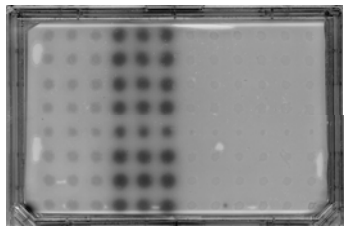
Direct colony assay



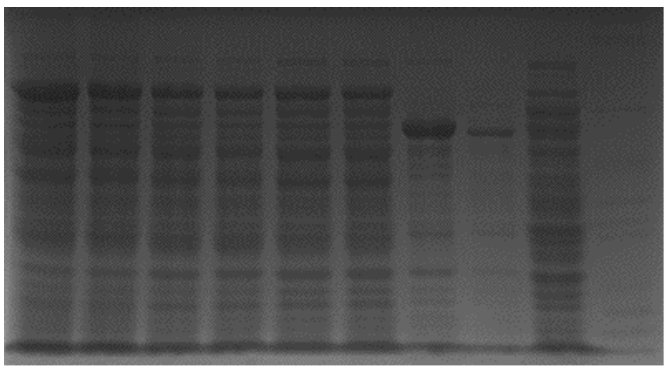
Microtiter plate assay

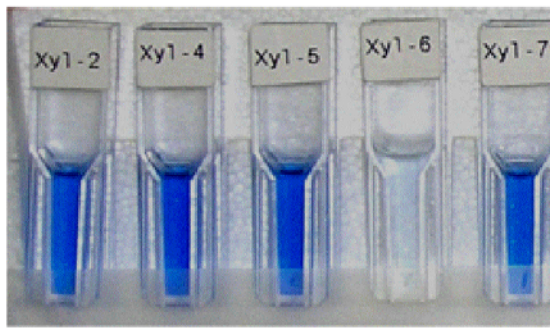


Membrane assay



Fusion xylosidase/arabinfuranosidase





Xylanase produced in transgenic tobacco

Summary

- Important to increase efficiency and reduce costs
- Spork and SAK approaches are feasible
- Directed evolution is a powerful tool
- Transgenic plants have great potential

- Significant progress made in HT screening and library making
- Active fusion enzyme generated
- Transgenic plant with high-levels of xylanase produced

- Collaborations with UK and USDA scientists are fruitful
- Thank you, KOEP, for the support
- Your support has led to an USDA/CSREES grant awarded to us (one of the objectives of the seed grant accomplished)